Identification of novel genes determining early adipogenesis in 3T3-L1 cells using conditioned differentiation medium

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Identification of novel genes determining early adipogenesis in 3T3-L1 cells using conditioned differentiation medium

Directed by Professor Jae-woo Kim

The Master's Thesis

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Yoonjeong Choi

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This certifies that the Master's Thesis of Yoonjeong Choi is approved.

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최윤정 드림

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Identification of novel genes determining early adipogenesis in 3T3-L1 cells using conditioned differentiation medium

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(Directed by Professor Jae-woo Kim)

Confluent 3T3-L1 preadipocytes initiate differentiation into adipocytes when treated with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI). After MDI induction, differentiating cells secrete various molecules into medium, and this medium is designated as conditioned medium (hereafter called CM). When CM was treated to 3T3-L1 preadipocytes, adipocyte differentiation was accelerated compared to when MDI alone was treated. In this regard, it was assumed that in the CM, there are some specific molecules secreted by adipocytes, which facilitate differentiation. On the basis of this assumption, this study was started to search for

novel genes which are expected to promote 3T3-L1 differentiation. To investigate candidate genes, RNA sequencing was performed using cells treated with MDI or CM. The RNA sequencing data were filtered out and 51 candidate genes were selected which are highly expressed in CM compared with MDI. In sequence, through confirming actual gene expression in 3T3-L1 cells and distribution in mouse fat tissue, three genes, Serpina3c, Serpina3n, and Adamts15 were selected. The result from RNA interference study aimed at these three genes, Serpina3c was finally selected as a new molecule expected to be related with adipocyte differentiation. Serpina3c, serine protease inhibitor A3C, belongs to Serpin superfamily. In 3T3-L1 cells, knockdown of Serpina3c resulted in the inhibition of mitotic clonal expansion. In addition, Serpina3c-knockdown cells showed that integrin α5 and ERK activation were decreased whereas AKT activation was increased. These results suggest that Serpina3c may play as a contributing factor in adipogenesis via IGF-1 signaling mediated by integrin α5.

Key words: adipogenesis, 3T3-L1, conditioned medium, Serina3c, integrin

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I. INTRODUCTION

Obesity is a world-wide disease and is associated with other metabolic disorders such as diabetes, hypertension and cardiovascular disease.[1] Up to date, there have been many efforts to conquer this problem, and studies about adipogenesis is important to understand mechanisms of obesity.[2] A number of molecular mechanisms that involved in adipocyte development had been elucidated, but many of researches were focused on the late stage of differentiation when the adipocyte specific genes are already expressed.[3] In this study, it was aimed to

identify novel genes which trigger adipogenesis in the early stage of differentiation using conditioned medium as a tool of screening.

Adipogenesis is a complex procedure to form new adipocytes from preadipocyte precursors.[4] 3T3-L1 preadipocyte cell line is a widely used *in vitro* model to study about molecular mechanisms underlying orchestrate adipocyte differentiation.[5] 3T3-L1 cells are committed stem cells and require adipogenic hormonal cocktail to initiate adipogenesis. When proliferating preadipocytes became confluent in culture dishes, they stop cell cycle and enter G_1 growth arrest steps.[6] Upon exposing hormonal cocktail (a mixture of 3-isobutyl-1-methylzanthine, dexamethasone, insulin, hereafter called MDI) and fetal bovine serum, growth arrested preadipocytes reenter the cell cycle and initiate differentiation via serial adipogenic gene expression such as CCAAT/enhancer binding protein β (C/EBPβ), CCAAT/enhancer binding protein α (C/EBPα) and Peroxisome proliferator-activated receptor (PPARγ).[5-7] This restarted cell cycle sustains during 24-48 hr and it is called mitotic clonal expansion. Mitotic clonal expansion is essential for 3T3-L1 differentiation, because selective inhibition of each steps of this process totally block adipogenesis.[6]

In the previous study, it was revealed that conditioned medium (hereafter called CM) which was collected from differentiating 3T3-L1 cells accelerates other preadipocytes differentiation as compared with MDI.[8] And surprisingly, mitotic clonal expansion which is critical for 3T3-L1 cells differentiation was skipped in CM-induced adipocytes. Accordingly, it was speculated that in the CM, there are some specific molecules facilitating differentiation. On that basis, this study was

started to search for novel genes which are expected to promote 3T3-L1 cells differentiation by using CM as a tool for screening.

After 2 days from induction by MDI, the cultured medium was collected and designated as CM. And then, CM was treated to other confluent preadipocytes to induce differentiation. These CM-treated adipocytes were harvested each 4 hr and 24 hr after induction. Equally, only MDI treated adipocytes were used as control groups. The harvested cells were subjected to perform RNA sequencing, to identify new genes involved in CM-controlled adipogenesis.

From the raw data of RNA sequencing, it was screened 51 candidate genes which were highly expressed in CM compared with MDI. In sequence, actual expression of these candidate genes in 3T3-L1 cells was confirmed. As a result, 3 genes, Sernpina3c, Serpina3n and Adamts15 were selected among the candidates. Conducting RNA interference assay, Serine protease inhibitor A3C (Serpina3c) was finally chosen as a candidate gene and I focused on its effect on adipocyte differentiation.

Knockdown of Serina3c by siRNA in 3T3-L1 showed substantial decrement in differentiation. Through further research, it is expected to identify the function of Serpina3c in 3T3-L1 adipocyte and this observation may afford a promising approach to understand adipogenesis.

II. MATERIALS AND METHODS

1. Cell culture and induction of differentiation

3T3-L1 preadipocytes were maintained Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 8 mg/ml biotin, supplemented with 10% heat-inactivated calf serum (Invitrogen) at 37°C, in an atmosphere of 90% air and 10% CO_2. To induce differentiation, 2-day postconfluent 3T3-L1 cells (designated day 0) were incubated in DMEM containing 10% fetal bovine serum (FBS, Invitrogen), 0.5 mM 3-isobutyl-1-methylzanthine (Sigma, St. Louis, MO, USA), 1 μM dexamethasone (Sigma), and 1 μg/ml of insulin (Roche, Penzberg, Germany) (designated as MDI) for 2 days. Cells were then cultured in DMEM containing 10% FBS and insulin for another 2 days, after which they were grown in DMEM containing FBS. For preparation of CM, 2-day postconfluent 3T3-L1 cells were induced by hormonal agents for 48 hr and cell-exposed medium was harvested.

2. Oil red-O staining

After differentiation, cells were washed once in phosphate-buffered saline (PBS) and fixed with 4% formalin in phosphate-buffered saline for 5 min, rinsed with distilled water. The staining solution was prepared by dissolving 0.5 g oil red-O (Sigma) in 100 ml of isopropanol; 6 ml of this solution was mixed with 4 ml of

distilled water, and filtered. The fixed with staining solution for 1 hr. The staining solution was removed and cells were rinsed twice with distilled water.

3. RNA isolation and PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis from 5 μg of total RNA was performed using SuperScript II reverse transcriptase (Invitrogen) primed by random hexamer primer. The transcript of Gpr64, Serpina3n, Adamts15, Btg3, Pde7b, Tgfa, Mia, Serpina3c, Fgfr2, Bcar3 and GAPDH were evaluated by PCR analysis. PCR was performed using the forward and reverse primers; 5'-TCAGT CTCAT CCCCT ACTCC A-3', 5'-TGAGC TCTGA TCGCT TCAGT-3' (Gpr64), 5'-CACTG TGGTG GAGCT GAAGT-3', 5'-TGTGG ACCAC CTGAG AGACT-3' (Serpina3n), 5'-GTGGC GGACG AGTCA ATG-3', 5'- GCATA GGTCC TGTCT GGTG AA-3' (Adamts15), 5'- GCTGC CGGTA TGGAG AGAAA-3', 5'-AATCT GGTAC ACGGG GCTTG-3' (Btg3), 5'-TTGAG AGGTG TGGCG AAGTC-3', 5'-GAGGG GCCTG CGGTA TAATC-3' (Pde7b), 5'-CTCTG CTAGC GCTGG GTATC-3', 5'-ATGGC TTGCT TCTTC TGGCT-3' (Tgfa), 5'-CGAGG AATGC AGCCA TCCTA T-3', 5'-CTCCC GGACA ATGCT ACTGG-3' (Mia), 5'-TGGCC TCCAT CAACA CTGAC-3', 5'-ATGGC TGAGC CTCTG TAGGA-3' (Serpina3c), 5'-GAAGG AGATC ACGGC TTCCC-3', 5'-CTCGG CCGAA ACTGT TACCT-3' (Fgfr2), 5'-AGTTT GCAAG CCTTC CCAGA-3', 5'-TGTCG TGGGG ATTTG GAGTG-3' (Bcar3), 5'-ACCAC AGTCC ATGCC ATCAC-3', 5'-TCCAC CACCC TGTTG CTGTA-3' (GAPDH).

4. RNA sequencing

To induce differentiation, confluent 3T3-L1 preadipocytes were treated MDI or CM respectively. After 4 hr and 24 hr, cells were harvested and total RNA was isolated with Trizol reagent (Invitrogen) according to manufacturer's instructions. Total RNA was commissioned to perform RNA sequencing (DNA Link, Seoul, Korea).

5. Western blot analysis and antibody

For protein analysis, cells were washed with cold PBS. Cells were then lysed directly in lysis buffer containing 1% sodium dodecyl sulfate (SDS) and 60 mM Tris-HCl, pH 6.8. The cell lysate was heated at 100°C and centrifuged for 5 min at 13,000 rpm and supernatants were frozen until further analysis. For western blotting, cell extracts were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blocked with blocking buffer (5% nonfat dry milk in Tris-buffered saline-tween 20, TBST) prior to incubation with antibody. Primary antibodies used for blotting were anti-C/EBPβ[9], anti-C/EBPα[9]. Anti-PPARγ, anti-α5, anti-α6, anti-pβ3 (Santa Cruz, CA, USA), anti-FLAG (Sigma), anti-GAPDH, anti-pERK, anti-ERK, anti-pAKT, anti-AKT, anti-pGSK3β, anti-GSKβ (Cell signaling, Danvers, MA, USA). After blocking filters were immunoblotted with each primary antibody for 2 hr at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 1 hr. Target proteins were visualized by

enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA).

6. FACS analysis

3T3-L1 cells were trypsinized and collected by centrifugation, washed with PBS, and fixed with 90% cold methanol. Fixed cells were washed with PBS and stained with 50 μg/ml propidium iodide and 100 μg/ml RNase A in PBS for 30 min in the dark. Labeled cells were analyzed using a FACS caliber low cytrometry system (BD Biosciences, San Jose, California, USA) and data were analyzed using ModeFit software.

7. Cell count assay

3T3-L1 cells were seeded at a density of 1.5×10^5 cells/well into 12-well plates. Cells were trypsinized and cell numbers were determined using an automated cell counter, ADAM (NanoEnTek), according to the manufacturer's instructions.

8. RNA interference (siRNA)

3T3-L1 preadipocytes were plated into 35 mm-dishes 18-24 hr prior to transfection. The following double-stranded stealth siRNA oligonucleotides (Santa Cruz) were used: mouse Serpina3C, set of two validated siRNA oligonucleotides (cat# sc-153358), mouse Serpina3n, set of three validated siRNA oligonucleotides (cat# sc-153364), mouse Adamts15, set of three validated siRNA oligonucleotides

(cat# sc-72444). Control oligonucleotides with comparable GC content were from Bioneer (Bioneer, Deajeon, Korea) (cat# SN-1013). For knockdown, cells were transfected with control or gene-specific siRNA at 50 nM in OPTI-MEM medium using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. The next day, the medium was replaced with fresh DMEM containing 10% calf serum and the cells were incubated for 24 hr before the induction of differentiation. Total RNA and protein extracts were prepared from the cells at the indicated time points, and RT-PCR and immunoblot analysis were performed. Oil red-O staining of Serpina3c, Serpina3n, Adamts15 were performed at day 7.

9. Transient transfection assay

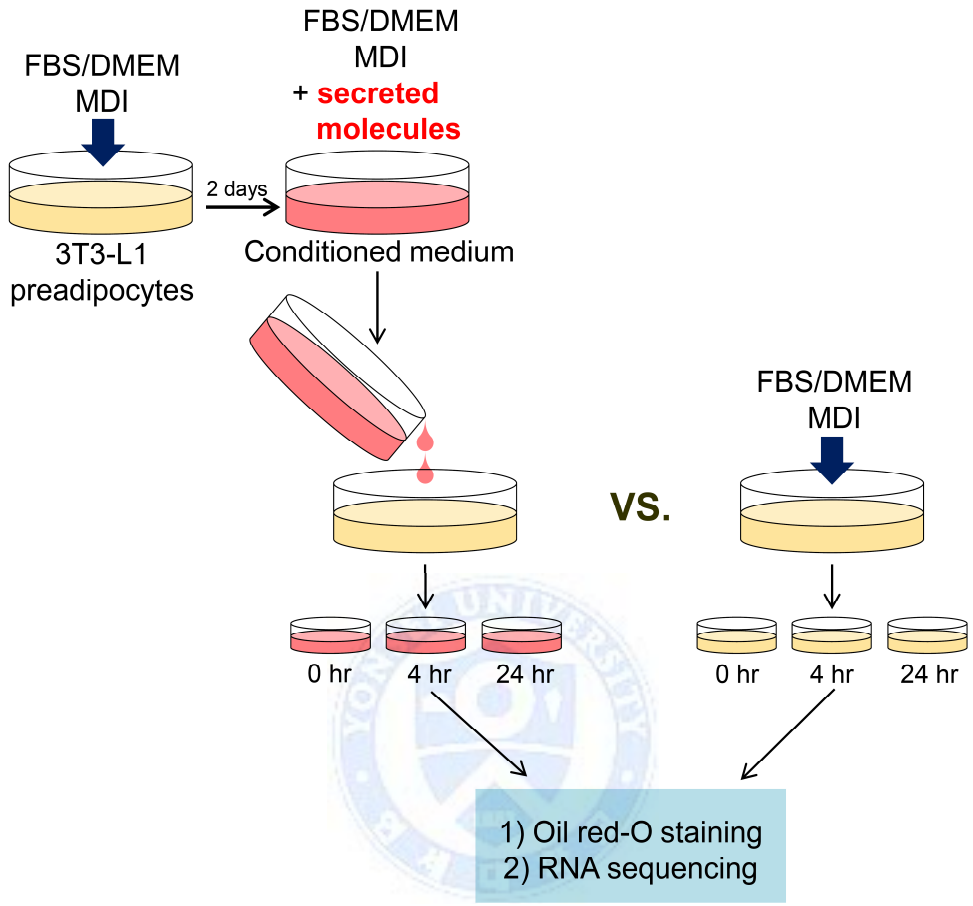
Serpina3c overexpression vector (pcDNA3.0-Serpina3c-FLAG) was generated by inserting whole open reading frame of mouse Serpina3c with C-terminal FLAG tag into pcDNA3.0 (Invitrogen). To maximize the transfection efficiency, microliter volume electrophoration of 3T3-L1 preadipocytes was performed with OneDrop MicroPorator MP-100 (Digital Bio, Seoul, Korea). The cells were trypsinized, washed with 1x PBS, and finally resuspended in 10 μl of resuspension buffer R and 0.5 μg of plasmid at a concentration of 200,000 cells per pipette. The cells were then microporated at 1,300 V, with a 20-ms pulse width, 2 pulses. Following microporation, the celle were seeded in 35-mm cell culture dished and placed at 37°C in a 10% CO_2-humidified atmosphere.

III. RESULT

1. Conditioned medium accelerated 3T3-L1 preadipocytes differentiation

In the first set of study, conditioned medium was prepared as follows. 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% calf serum until the cells were confluent in the culture dish and had stopped the cell cycle. After 2 days from confluency, preadipocytes were induced to differentiate by MDI. On day 2 of the differentiation, cell-exposed medium was collected and designated as CM which contains secreted molecules from differentiating adipocytes as well as MDI (Fig. 1A). To compare the effect of CM on 3T3-L1 differentiation, growth arrested 3T3-L1 preadipocytes were treated with either MDI or CM. As previously observed,[8] the preadipocytes treated with CM accelerated terminal differentiation evidenced by oil red-O staining (Fig. 1B).

A


FBS/DMEM
MDI
3T3-L1
preadipocytes
2 days
FBS/DMEM
MDI
+ secreted
molecules
Conditioned medium
VS.
FBS/DMEM
MDI
0 hr
4 hr
24 hr
0 hr
4 hr
24 hr
1) Oil red-O staining
2) RNA sequencing

B

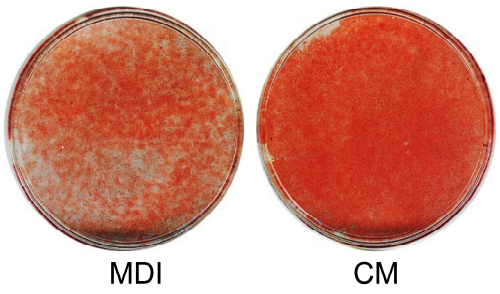

MDI
CM

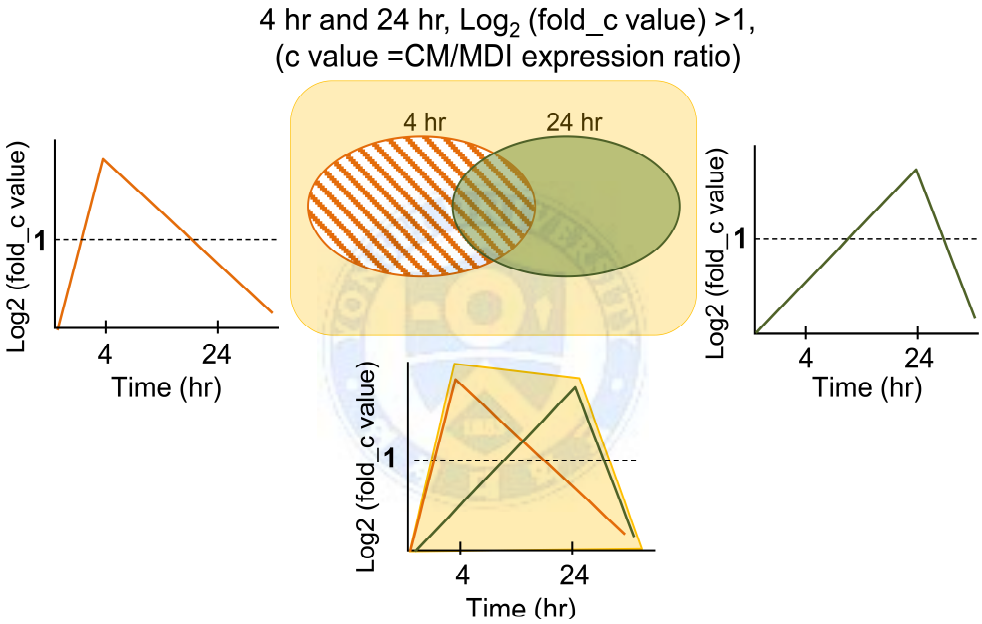
Figure 1. Conditioned medium and its effect on adipogenesis. CM-treated 3T3-L1 preadipocytes accelerated terminal differentiation. (A) A schematic model of CM preparation using a 3T3-L1 preadipocyte differentiation system. Confluent and growth arrested 3T3-L1 preadipocytes were induced differentiation by a treatment of hormonal cocktail, called MDI. After 2 days, cell-exposed medium containing secreted molecules as well as MDI was collected and designated as CM. This collected CM is treated to other preadipocytes to induce differentiation. (B) Confluent 3T3-L1 preadipocytes were treated with MDI or CM. The degree of differentiation was visualized by oil red-O staining on day 5.

2. Genes highly expressed in CM-facilitated adipocyte differentiation in early steps of adipogenesis

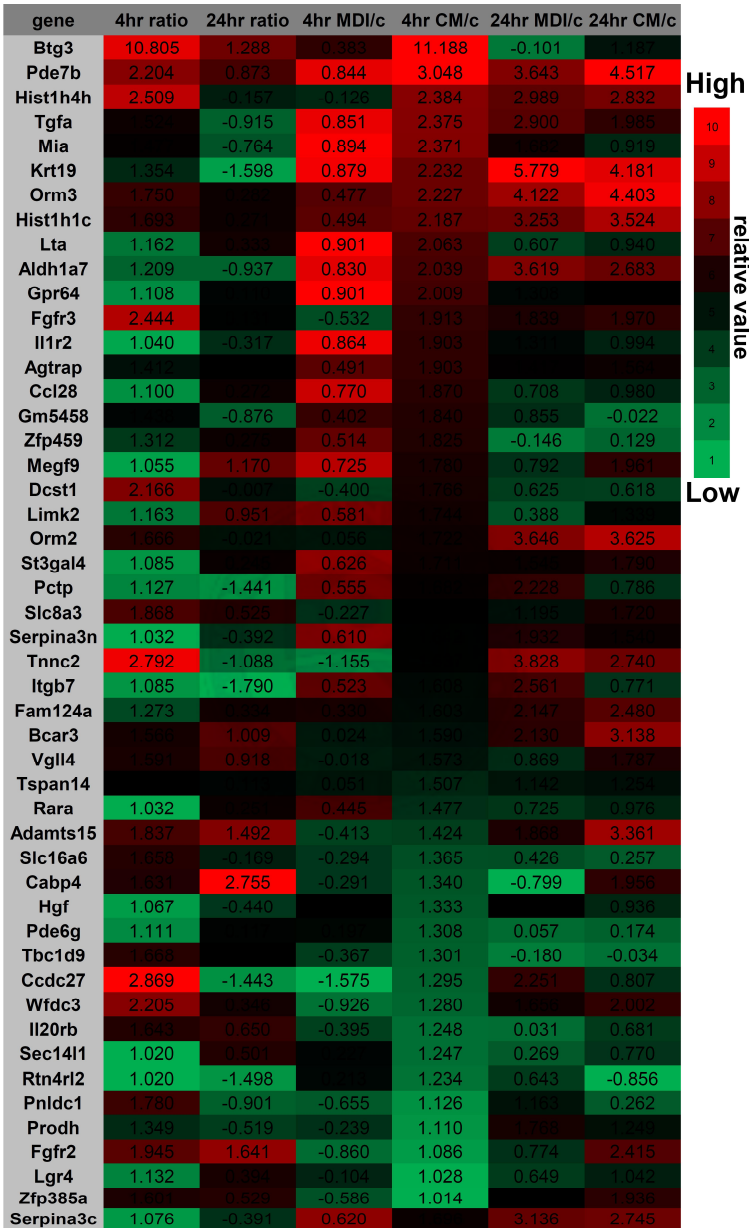
CM facilitated terminal differentiation in 3T3-L1 preadipocytes. Accordingly, it was speculated that in CM, there might be some specific molecules secreted from adipocytes. These secreted molecules may trigger the expression of unknown genes involved in adipogenesis. To find out this, it was searched for novel genes highly expressed in CM compared with MDI.

Differentiating cells were harvested after 4 hr and 24 hr from induction, and total RNA was requested to perform RNA sequencing. Among the RNA sequencing data, genes showing Log_2 (fold changes in CM/MDI) above 1 were selected. In other words, genes which were expressed more than two-fold higher in CM were chosen (Fig. 2A). In addition, only up-regulated genes after induction were chosen. Consequently, 51 genes meeting these criteria were selected. (Fig. 2B). This RNA sequencing data was integrated with other microarray data previously conducted on various adipocyte types and mouse fat tissues. On that basis, 10 candidate genes were selected considering the function of genes and coding proteins (Table 1).

A


4 hr and 24 hr, Log2 (fold_c value) >1,
(c value =CM/MDI expression ratio)
4 hr
24 hr
Log2 (fold_c value)
1
4
24
Time (hr)
Log2 (fold_c value)
1
4
24
Time (hr)
Log2 (fold_c value)
1
4
24
Time (hr)

B



gene	4hr ratio	24hr ratio	4hr MDI/c	4hr CM/c	24hr MDI/c	24hr CM/c
Btg3	10.805	1.288	0.383	11.188	-0.101	1.187
Pde7b	2.204	0.873	0.844	3.048	3.643	4.517
Hist1h4h	2.509	0.157	0.126	2.384	2.989	2.832
Tgfa	1.934	-0.915	0.851	2.375	2.900	1.985
Mia	1.507	-0.764	0.894	2.371	1.882	0.919
Krt19	1.354	-1.598	0.879	2.232	5.779	4.181
Orm3	1.750	0.282	0.477	2.227	4.122	4.403
Hist1h1c	1.693	0.271	0.494	2.187	3.253	3.524
Lta	1.162	0.333	0.901	2.063	0.607	0.940
Aldh1a7	1.209	-0.937	0.830	2.039	3.619	2.683
Gpr64	1.108	0.793	0.901	2.009	1.839	1.970
Fgfr3	2.444	1.387	-0.532	1.913	1.839	1.970
Il1r2	1.040	-0.317	0.864	1.903	1.311	0.994
Agtrap	1.412	0.491	0.491	1.903	1.311	0.994
Ccl28	1.100	0.670	0.770	1.870	0.708	0.980
Gm5458	2.723	-0.876	0.402	1.840	0.855	-0.022
Zfp459	1.312	0.273	0.514	1.825	-0.146	0.129
Megf9	1.055	1.170	0.725	1.780	0.792	1.961
Dcst1	2.166	-0.007	-0.400	1.768	0.625	0.618
Limk2	1.163	0.951	0.581	1.744	0.388	0.788
Orm2	1.666	0.021	0.056	1.722	3.646	3.625
St3gal4	1.085	0.705	0.626	1.711	1.695	1.790
Pctp	1.127	-1.441	0.555	1.683	2.228	0.786
Slc8a3	1.868	0.525	-0.227	1.677	1.195	1.720
Serpina3n	1.032	-0.392	0.610	1.642	1.932	1.981
Tnnc2	2.792	-1.088	-1.155	1.641	3.828	2.740
Itgb7	1.085	-1.790	0.523	1.609	2.561	0.771
Fam124a	1.273	0.334	0.338	1.603	2.147	2.480
Bcar3	1.566	1.009	0.024	1.590	2.130	3.138
Vgll4	1.591	0.918	-0.018	1.573	0.869	1.787
Tspan14	1.456	0.405	0.051	1.507	1.142	1.264
Rara	1.032	0.763	0.445	1.477	0.725	0.976
Adamts15	1.837	1.492	-0.413	1.424	1.868	3.361
Slc16a6	1.658	-0.169	-0.294	1.365	0.426	0.257
Cabp4	1.631	2.755	-0.291	1.340	-0.799	1.956
Hgf	1.067	-0.440	0.000	1.333	0.000	0.936
Pde6g	1.111	0.000	0.000	1.308	0.057	0.174
Tbc1d9	1.668	0.000	-0.367	1.301	-0.180	-0.034
Ccdc27	2.869	-1.443	-1.575	1.295	2.251	0.807
Wfdc3	2.205	0.346	-0.926	1.280	1.696	2.002
Il20rb	1.643	0.650	-0.395	1.248	0.031	0.681
Sec14l1	1.020	0.501	0.000	1.247	0.269	0.770
Rtn4rl2	1.020	-1.498	0.000	1.234	0.643	-0.856
Pnldc1	1.780	-0.901	-0.655	1.126	1.163	0.262
Prodh	1.349	-0.519	-0.239	1.110	1.768	1.249
Fgfr2	1.945	1.641	-0.860	1.086	0.774	2.415
Lgr4	1.132	0.394	-0.104	1.028	0.649	1.042
Zfp385a	1.601	0.529	-0.586	1.014	0.000	1.936
Serpina3c	1.076	-0.391	0.620	0.000	3.136	2.745

Figure 2. A schematic model of the criteria for screening and relative gene expression in CM. (A) A schematic model of the criteria for screening in RNA sequencing data. Either CM or MDI were treated to 3T3-L1 cells and fold changes of relative gene expression was measured by RNA sequencing. After 4 hr from induction, genes showing Log_2 (fold changes in CM/MDI) above 1 were selected. Equally, after 24 hr, genes were selected as above. The union of these two groups, 4 hr and 24 hr, were chosen as candidate genes. (B) Visualization of the gene expression level. Red means high and green means low expression. All number means fold changes.

Table 1. Fold changes of candidate genes

gene	4hr ratio	24hr ratio	4hr MDI/c	4hr CM/c	24hr MDI/c	24hr CM/c	WAT	SAT-B	SAT-I	BAT	L1	A33
Btg3	10.805	1.288	0.383	11.188	-0.101	1.187						
Pde7b	2.204	0.873	0.844	3.048	3.643	4.517						
Hist1h4h	2.509	-0.157	-0.126	2.384	2.989	2.832	2.912	2.4	2.854	3.355	3.505	3.842
Tgfa	1.524	-0.915	0.851	2.375	2.9	1.985	6.37	6.829	6.541	6.077	6.099	5.919
Mia	1.477	-0.764	0.894	2.371	1.682	0.919	5.83	5.208	5.126	5.032	5.63	5.44
Krt19	1.354	-1.598	0.879	2.232	5.779	4.181	7.751	6.319	7.453	6.018	6.472	6.21
Orm3	1.75	0.282	0.477	2.227	4.122	4.403	9.289	7.573	10.011	10.339	9.239	11.181
Hist1h1c	1.693	0.271	0.494	2.187	3.253	3.524	10.534	10.225	10.55	10.642	11.064	12.127
Lta	1.162	0.333	0.901	2.063	0.607	0.94	5.961	7.282	7.039	5.834	6.998	6.19
Aldh1a7	1.209	-0.937	0.83	2.039	3.619	2.683	9.098	7.985	9.134	9.216	10.254	10.433
Gpr64	1.108	0.11	0.901	2.009	1.308	1.418	10.807	8.094	9.05	6.372	8.436	10.799
Fgfr3	2.444	0.131	-0.532	1.913	1.839	1.97	6.428	6.1	6.192	6.238	6.641	6.659
Il1r2	1.04	-0.317	0.864	1.903	1.311	0.994	5.665	8.877	7.996	6.839	5.272	5.564
Agtrap	1.412	0.147	0.491	1.903	1.417	1.564	7.65	7.864	7.613	7.668	8.433	8.088
Gm5458	1.438	-0.876	0.402	1.84	0.855	-0.022	7.499	7.056	6.576	7.058	6.409	6.81
Zfp459	1.312	0.275	0.514	1.825	-0.146	0.129	5.298	5.735	5.178	4.561	5.142	4.91
Megf9	1.055	1.17	0.725	1.78	0.792	1.961	9.176	8.646	9.361	10.018	9.067	9.449
Dcst1	2.166	-0.007	-0.4	1.766	0.625	0.618	7.69	6.621	6.386	6.043	6.129	6.301
Limk2	1.163	0.951	0.581	1.744	0.388	1.339	7.337	7.421	7.22	7.707	8.35	7.699
Orm2	1.666	-0.021	0.056	1.722	3.646	3.625	6.563	5.936	7.98	6.722	8.079	12.35
St3gal4	1.085	0.245	0.626	1.711	1.545	1.79	8.656	8.453	8.348	7.935	9.543	9.02
Pctp	1.127	-1.441	0.555	1.682	2.228	0.786	8.153	7.913	8.383	9.499	7.475	7.244
Slc8a3	1.868	0.525	-0.227	1.642	1.195	1.72	5.395	5.401	5.478	5.694	5.972	5.621
Serpina3n	1.032	-0.392	0.61	1.642	1.932	1.54	12.628	11.975	12.419	8.808	10.305	8.551
Tnnc2	2.792	-1.088	-1.155	1.637	3.828	2.74	7.244	7.578	9.56	8.351	7.224	6.949
Itgb7	1.085	-1.79	0.523	1.608	2.561	0.771	6.595	8.65	8.057	6.221	6.366	5.702
Bcar3	1.566	1.009	0.024	1.59	2.13	3.138	9.612	8.993	8.891	7.842	9.188	9.483
Tspan14	1.456	0.113	0.051	1.507	1.142	1.254	10.324	10.351	10.263	10.236	10.443	11.039
Rara	1.032	0.251	0.445	1.477	0.725	0.976	7.807	8.322	8.17	7.561	9.149	9.605
Adamts15	1.837	1.492	-0.413	1.424	1.868	3.361						
Slc16a6	1.658	-0.169	-0.294	1.365	0.426	0.257	7.199	7.948	7.65	6.51	6.89	6.144
Cabp4	1.631	2.755	-0.291	1.34	-0.799	1.956	7.17	6.78	7.634	6.128	7.7	7.29
Hgf	1.067	-0.44	0.266	1.333	1.376	0.936	7.566	7.272	7.492	6.834	6.885	7.977
Pde6g	1.111	0.117	0.197	1.308	0.057	0.174	6.45	6.471	6.388	6.728	6.641	6.816
Tbc1d9	1.668	0.146	-0.367	1.301	-0.18	-0.034	7.544	8.039	7.703	7.217	6.136	6.285
Ccdc27	2.869	-1.443	-1.575	1.295	2.251	0.807	5.425	5.436	5.322	4.893	5.534	5.599
Sec14l1	1.02	0.501	0.227	1.247	0.269	0.77	9.338	8.996	8.921	9.324	10.44	9.414
Prodh	1.349	-0.519	-0.239	1.11	1.768	1.249	8.578	7.549	8.611	8.085	9.384	7.235
Fgfr2	1.945	1.641	-0.86	1.086	0.774	2.415						
Serpina3c	1.076	-0.391	0.620	1.696	3.136	2.745	12.402	11.671	12.117	9.457	9.021	7.193

Integrated data with RNA sequencing (4 hr ratio, 24 hr ratio, 4 hr MDI/c, 4hr CM/c, 24 hr MDI/c, 24 hr CM/c) and microarray (WAT, SAT-B, SAT-I, BAT, L1, A33). 4 hr ratio, fold changes of 4hr CM/ 4 hr MDI; 24 hr ratio, fold changes of 24 hr CM/ 24 hr MDI; c, 0hr control; WAT, C57BL/6 mouse epididymal fat; SAT-B, mouse belly fat; SAT-I, mouse inguinal fat; L1, 3T3-L1 adipocytes; A33, A33 adipocytes.

3. CM-induced 3T3-L1 adipocytes showed different expression patterns compared with MDI

After selection of 10 candidates, actual expression of these genes in 3T3-L1 cells was confirmed. During the first 6 hr of CM or MDI exposure, expression of Adamts15 and Serpina3c were increased in CM (Fig. 3A). During the 5 days of induction, expression of Adamts15 and Serpina3c was induced earlier in CM than in MDI. Expression of Serpina3n was decreased earlier in CM compared with MDI (Fig. 3B). The short-term expression during 6 hr means immediate expressional response in 3T3-L1 cells against CM. And the long-term expression during 5 days means the changes of gene expression by CM after mitotic clonal expansion.

By comparing these different expression patterns in CM versus MDI, three genes, Serpina3c, Serpina3n, and Adamts15, were finally selected and researched further. In this study, although only these three genes were chosen as subjects, the other genes are also strong candidates expected to be involved in adipogenesis.

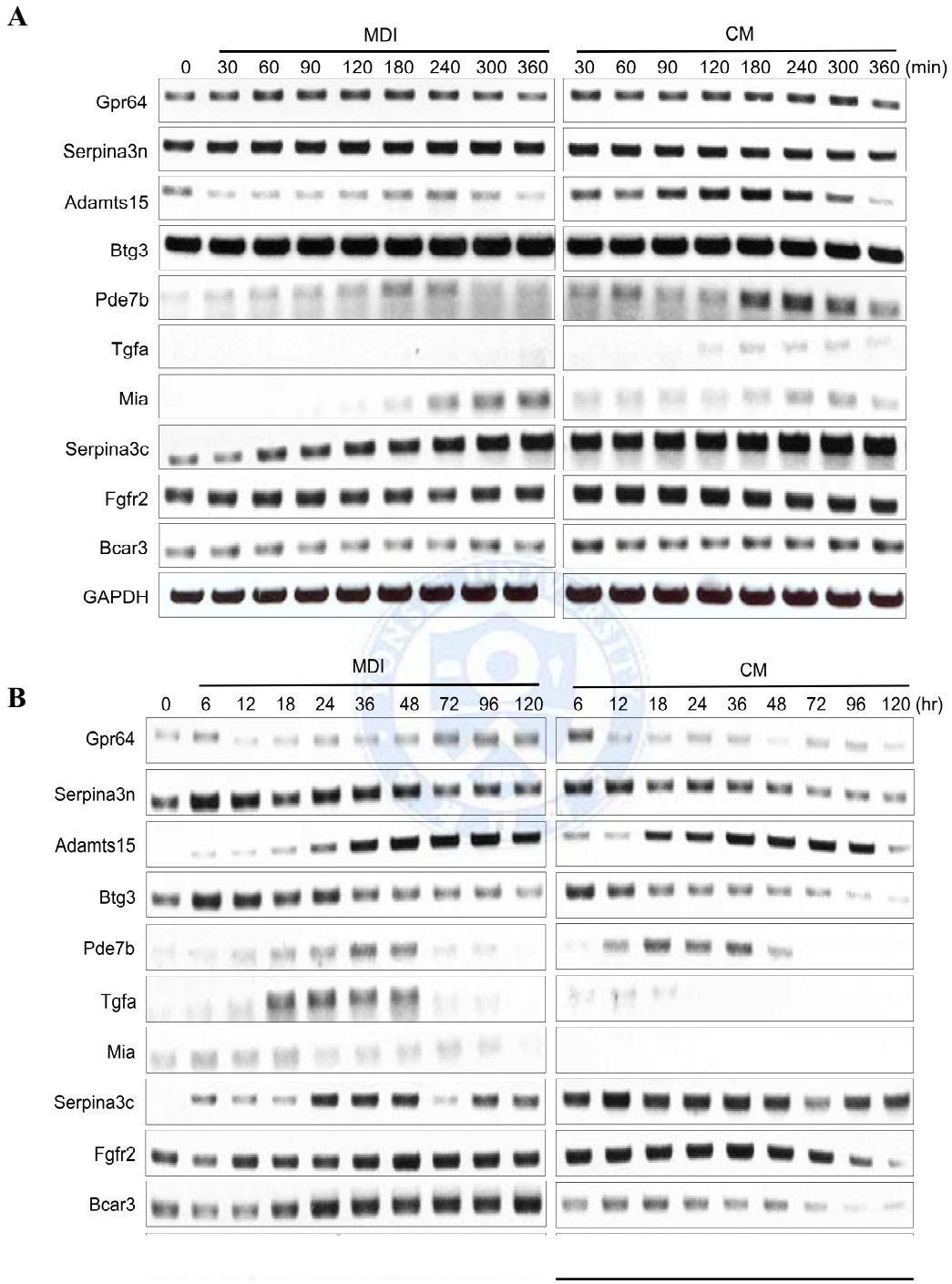

A
MDI
CM
0 30 60 90 120 180 240 300 360
30 60 90 120 180 240 300 360 (min)
Gpr64
Serpina3n
Adamts15
Btg3
Pde7b
Tgfa
Mia
Serpina3c
Fgfr2
Bcar3
GAPDH
B
MDI
CM
0 6 12 18 24 36 48 72 96 120
6 12 18 24 36 48 72 96 120 (hr)
Gpr64
Serpina3n
Adamts15
Btg3
Pde7b
Tgfa
Mia
Serpina3c
Fgfr2
Bcar3

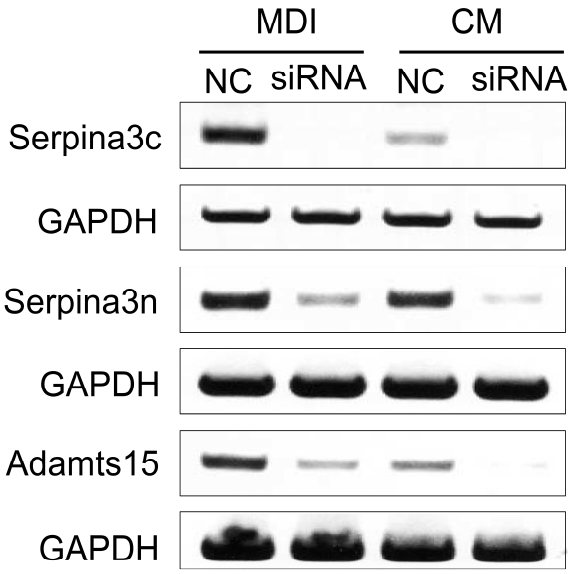
Figure 3. Expression patterns of selected genes in 3T3-L1 adipocytes. By comparing different expression patterns in CM versus MDI, three genes, Serpina3c, Serpina3n, and Adamts15, were finally selected. (A), (B) Cell lysates were prepared at various times after adipogenic induction, and subjected to RT-PCR for Gpr64, Serpina3n, Adamts15, Btg3, Pde7b, Tgfa, Mia, Serpina3c, Fgfr2, Bcar3, and GAPDH.

4. Serpina3c was expressed in mouse fat tissue and knockdown of Serpina3c affected adipocyte differentiation

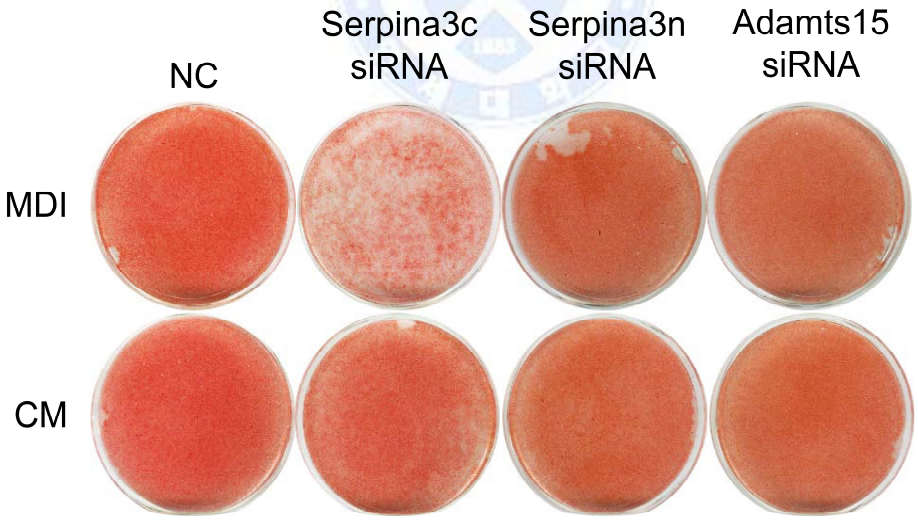
Tissue distribution of Serpina3c, Serpina3n, and Adamts15 revealed that Serpina3c and Serpina3n were significantly high in mouse adipose tissue and, to a lesser or equal degree, in brain, lung and liver, compared to other tissues (Fig. 4). This suggests that Serpina3c and Serpina3n may contribute to forming adipose tissue *in vivo*. To examine whether Serpina3c, Serpina3n, and Adamts15 affect adipogenesis, these genes were knocked down in 3T3-L1 cells using siRNA. As a result, Serpina3c-knockdown cells were induced decrement in differentiation evidenced by oil red-O staining. On the other hand, Serpina3n and Adamts15 affected differentiation insignificantly (Fig. 5B). Suppressed differentiation by Serpina3c-knockdown was also confirmed by microscope images (Fig. 5C). These results indicated that Serpina3c is the most powerful candidate gene expected to facilitate adipogenesis among the CM-used RNA sequencing data.

Figure 4. Expression of Serpina3, Serpina3n, and Adamts15 in various mouse tissues. Tissue distribution of Serpina3c, Serpina3n, and Adamts15 genes by using RT-PCR. Mouse tissues were extracted from C57BL/6 mice fed a chow diet.

A


MDI
CM
NC
siRNA
NC
siRNA
Serpina3c
GAPDH
Serpina3n
GAPDH
Adamts15
GAPDH

B


NC
Serpina3c siRNA
Serpina3n siRNA
Adamts15 siRNA
MDI
CM

C

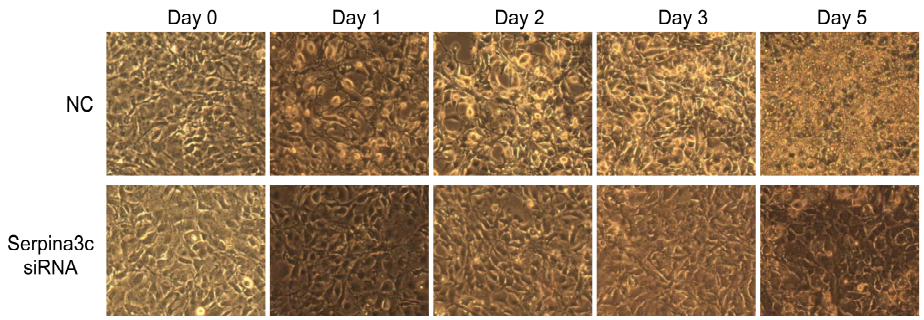


Figure 5. Knockdown of Serpina3c, Serpina3n and Adamts15 in 3T3-L1 cells. Serpina3c is the most powerful candidate gene expected to facilitate adipogenesis. (A) Knockdown of Serpina3c, Serpina3n, and Adamts15 mRNA in 3T3-L1 cells was assessed by RT-PRC. (B) Oil red-O staining of adipocytes with negative control (NC) and Serpina3c, Serpina3n, Adamts15 knockdown at day 8. (C) Microscope images of differentiating 3T3-L1 cells with negative control (NC) and Serpina3c knockdown.

5. Serpina3c is a secretory protein and has no functional relationship with Serpina3n in adipocyte differentiation.

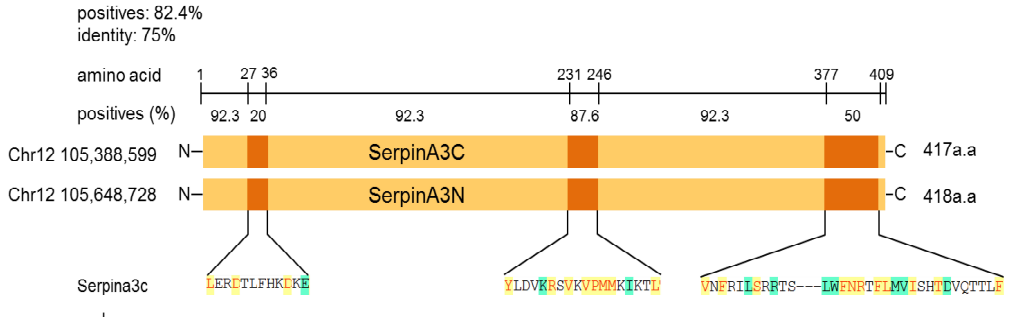
From the RNA interference assay, only Serpina3c-knockdown resulted in the inhibition of adipocyte differentiation (Fig. 5B). However, Serpina3c and Serpina3n have very similar sequence homology and protein structures. Besides, Serpina3c and Serpina3n exist on the same chromosome in the near distance (Fig. 6A). Taking one with another, there are some possibilities that Serpina3c and Serpina3n may have functional relationship associated with adipogenesis. To find out this, double knockdown of Sernpina3c and Serpina3n was conducted in 3T3-L1 cells (Fig. 6B). As a result, there was no functional relationship between Serpina3c and Serpina3n in adipogenesis, but only Serpina3c had effect on 3T3-L1 cells differentiation (Fig. 6C).

Serpina3c is a simplified form of serine protease inhibitor A3C and its alternative name is alpha 1 proteinase inhibitor antitrypsin. Serpina3c is a member of Serpin superfamily and is generally known to be related with emphysema, chronic fatigue, and thromboembolic disorders (Table 2).[10,11]

Serpina3c is predicted as a secreted protein with signal peptide on its N-terminal sequence (Fig. 7A). To generate *in vitro* Serpina3c overexpressing vector, open reading frame of mouse Serpina3c was inserted into pcDNA3.0. Because of the signal peptide, FLAG tag was inserted into C-terminal of Serpina3c. When FLAG-tagged Serpina3c was transfected into HEK 293T cells, secreted Serpina3c was found in the medium (Fig. 7B). From these data, it can be assumed that

secreted Serpina3c in the medium may take unknown actions in extracellular spaces during adipogenesis.

A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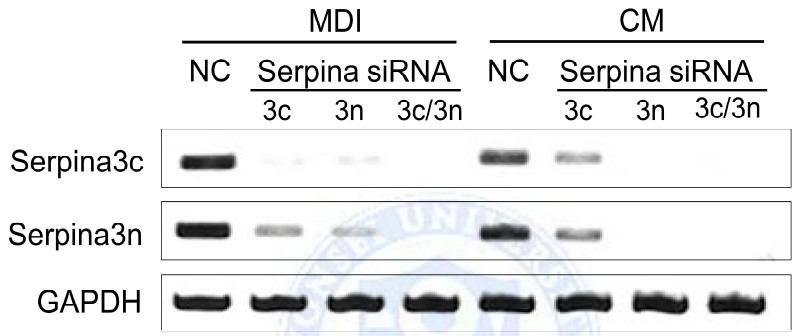
identity: 75%
amino acid
1
27 36
231 246
377
409
positives (%)
92.3 20
92.3
87.6
92.3
50
Chr12 105,388,599
N–
SerpinA3C
–C
417a.a
Chr12 105,648,728
N–
SerpinA3N
–C
418a.a
Serpina3c
LERLTLFHKLKE
YLDVKRSVKVPMMKIKTL
VNFRIISRRTS---LWFNRTFLMVISHTLVQTTLF

B


MDI
CM
NC
Serpina siRNA
NC
Serpina siRNA
3c
3n
3c/3n
3c
3n
3c/3n
Serpina3c
Serpina3n
GAPDH

C

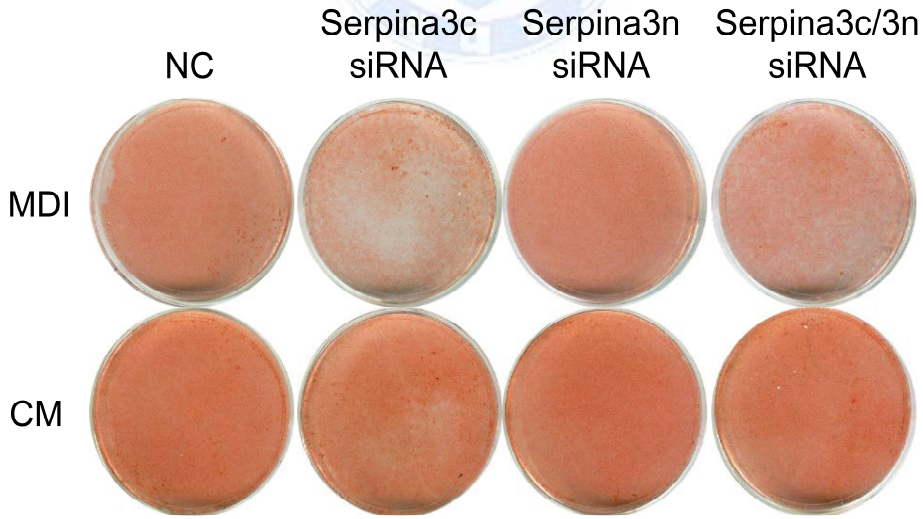
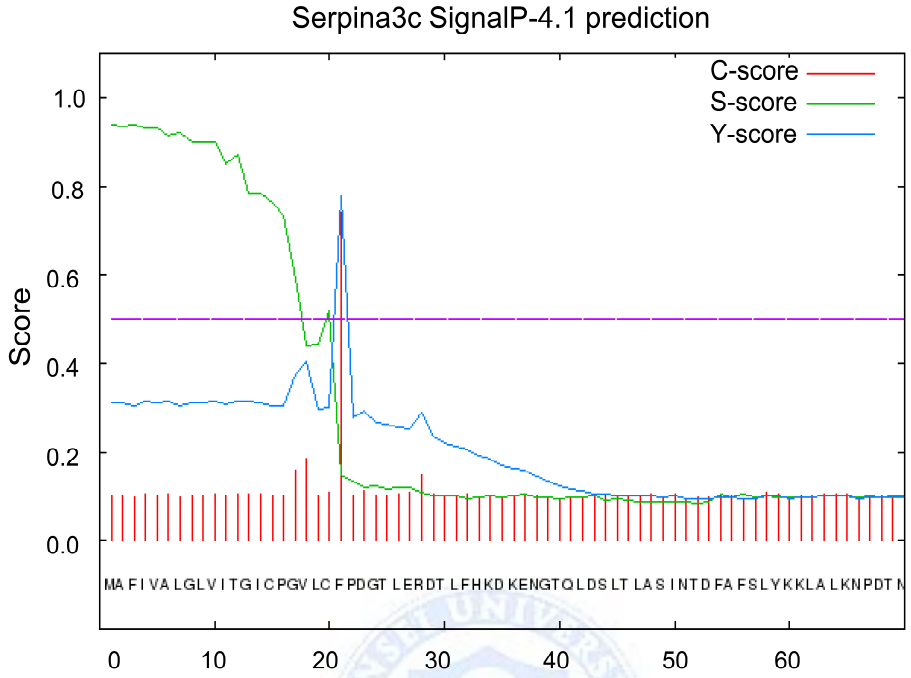

NC
Serpina3c siRNA
Serpina3n siRNA
Serpina3c/3n siRNA
MDI
CM

Figure 6. Double knockdown of Serpina3c and Serpina3n. There was no functional relationship between Serpina3c and Serpina3n in adipogenesis, but only Serpina3c had effect on 3T3-L1 cells differentiation. (A) A schematic model of Serpina3c and Serpina3n amino acid sequence. Sequences were aligned using Vector NTI 7.0. (B) Double knockdown of Serpina3c and Serpina3n was assessed by RT-PCR. (C) Oil red-O staining of adipocytes with negative control (NC) and single knockdown.

Table 2. Description of Serpin superfamily

Clade name[11]	Serpin gene name	Subtype	Localization[10]	Involvement in disease
Alpha 1 proteinase inhibitor antitrypsin	SerpinA	A1-13	Extracellular	Deficiency result in emphysema, chronic fatigue, hypothyroidism and thromboembolic disease[12-14] Polymerization and retention in the ER results in cirrhosis[15,16] Linked to angioedema, hypertension[17]
ov Serpins	SerpinB	B1-13	Intracellular	Down regulation linked to tumor progression and overall prognosis[18] Linked to IgA nephropathy
Antithrombin	SerpinC	C1	Extracellular	Deficiency results in thrombosis[19]
Heparin cofactor	SerpinD	D1	Extracellular	Increasing thrombotic risk[20]
Nexin/plasmino gene activator inhibitor 1	SerpinE	E1-3	Extracellular	Abnormal bleeding[21]
Alpha 2 antiplasmin pigment epithelium derived factor	SerpinF	F1-2	Extracellular	Unrestrained fibrinolytic activity, bleeding[22]
C1 inhibitor	SerpinG	G1	Not characterized	Angioedema[23]
Heat shock protein	SerpinH	H1	Not characterized	
Neuroserpin I	SerpinI	I1-2	Extracellular	Polymerization results in dementia[24]

A


Serpina3c SignalP-4.1 prediction
C-score
S-score
Y-score
Score
1.0
0.8
0.6
0.4
0.2
0.0
MA F I VA LGLV I TG I CPGV LC F PDGT LERDT LFHKD KENGTQLDS LT LA S I NT D FA F SLYKKLA LKNPDT N
0
10
20
30
40
50
60

B

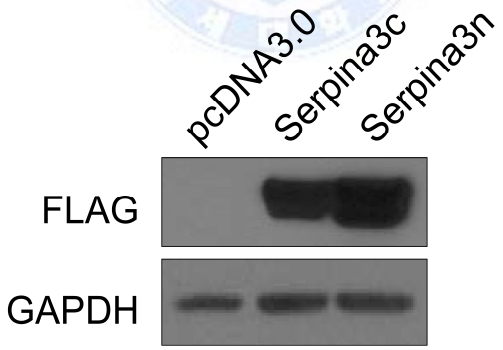

pcDNA3.0
Serpina3c
Serpina3n
FLAG
GAPDH

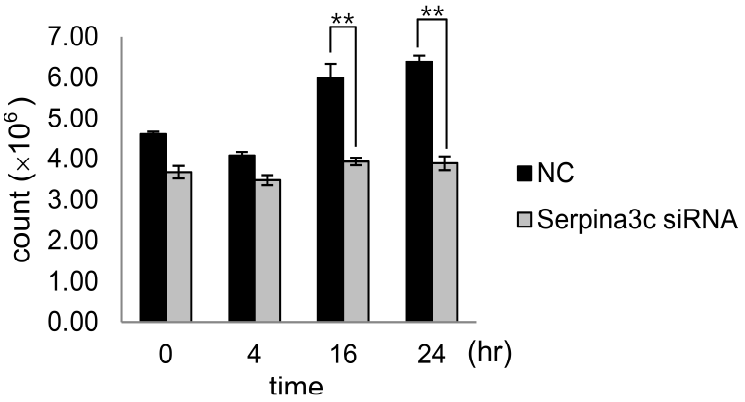
Figure 7. Serpina3c is a secretory protein. (A) Amino acid sequence of Serpina3c was analyzed by SignalP-4.1 prediction (www.cbc.dk/services/SignalP/). (B) Overexpression vector was generated by inserting mouse Serpina3c into pcDNA3.0 with C-terminal FLAG-tag. This overexpression vector was transfected into HEK293T cells and cell-exposed medium was collected after two days from transfection. Cell-exposed medium was precipitated by cold acetone and western blot analysis was conducted against FLAG antibody to detect secreted Serpina3c.

6. Knockdown of Serpina3c inhibited mitotic clonal expansion

The mitotic clonal expansion of 3T3-L1 is involved in the sequential expression of C/EBPβ, C/EBPα, and PPARγ, which are adipocyte-specific genes. To elucidate how Serpina3c is involved in adipogenesis, it was assessed whether Serpina3c is associated with mitotic clonal expansion.

During mitotic clonal expansion, cell numbers increase 2-3 folds in 24-48 hr. However, in si-Serpina3c knockdown cells, cell numbers did not increase after MDI induction (Fig. 8A). These results were also confirmed by FACS analysis. Whereas control cells reentered the cell cycle successfully, si-Serpina3c cells were arrested at G_1 and did not reenter the cell cycle. (Fig. 8B). These data suggest that deficiency of Serpina3c prevents mitotic clonal expansion resulting in G_1-S transition.

A


7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
count (×10^6)
**
**
0
4
16
24
(hr)
time
NC
Serpina3c siRNA

B

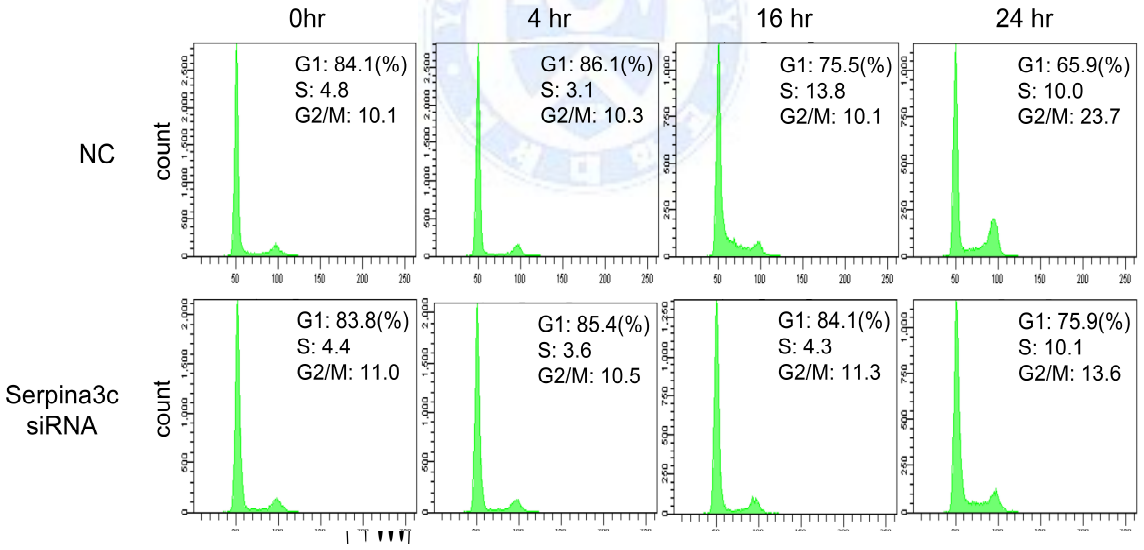

0hr
4 hr
16 hr
24 hr
NC
Serpina3c siRNA
count
G1: 84.1(%)
S: 4.8
G2/M: 10.1
G1: 86.1(%)
S: 3.1
G2/M: 10.3
G1: 75.5(%)
S: 13.8
G2/M: 10.1
G1: 65.9(%)
S: 10.0
G2/M: 23.7
G1: 83.8(%)
S: 4.4
G2/M: 11.0
G1: 85.4(%)
S: 3.6
G2/M: 10.5
G1: 84.1(%)
S: 4.3
G2/M: 11.3
G1: 75.9(%)
S: 10.1
G2/M: 13.6

Figure 8. Serpina3c is required for mitotic clonal expansion during adipogenesis. 3T3-L1 cells were transfected with negative control (NC), or Serpina3c siRNA, and then differentiated using IBMX, insulin, and dexamethasone (MDI). (A) Cell proliferation determining by cell counting at 4 hr, 16 hr, and 24 hr after induction. This is the representative result of 4 independent experiments. (B) DNA contents were analyzed by flow cytometry at the indicated time points. This is the representative result of 2 independent experiments.

7. Serpina3c was involved in adipogenesis via integrin-medigated IGF-1 pathway

Previous results show that Serpina3c is required for mitotic clonal expansion, but the underlying molecular mechanisms are unclear.

The mitotic clonal expansion of 3T3-L1 cells is involved in activation of C/EBPβ protein by MAPK and glycogen synthase kinase 3β (GSKβ).[7,25] These regulators are required for the expression of the two principal adipogenic factors, C/EBPα and PPARγ.[3,4]

Although there are little informations about Serpina3c, Serpina3 clade has been known as an inhibitor of cathepsin G.[10,11] In addition, some researchers showed that cathepsin G is involved in integrin clustering in immune cells.[26,27] Integrins form a complex with IGF-1 receptor and arouse signal transduction of downstream signaling.[27,28] Therefore, it was hypothesized that Serpina3c may be involved in adipogenesis via integrin-mediated IGF-1 signaling (Fig. 9A).

To examine how Serpina3c affects integrins, Serpina3c was knocked down with siRNA in 3T3-L1 cells and three types of integrins were confirmed by western blot analysis (Fig. 9B). Interestingly, integrin α5 and phosphorylated β3 were diminished in Serpina3c-knockdown cells. But α6 was unaffected by Serpina3c. In 3T3-L1 cells, α5 and β3 form heterodimer and interact with IGF-1 receptor.[29] When the ligands bind, β3-Tyr759 is phosphorylated and this phosphorylation leads to Src binding.[30,31] As above results suggest, ERK activation was diminished in Serpina3c-knockdown cells (Fig. 9B).

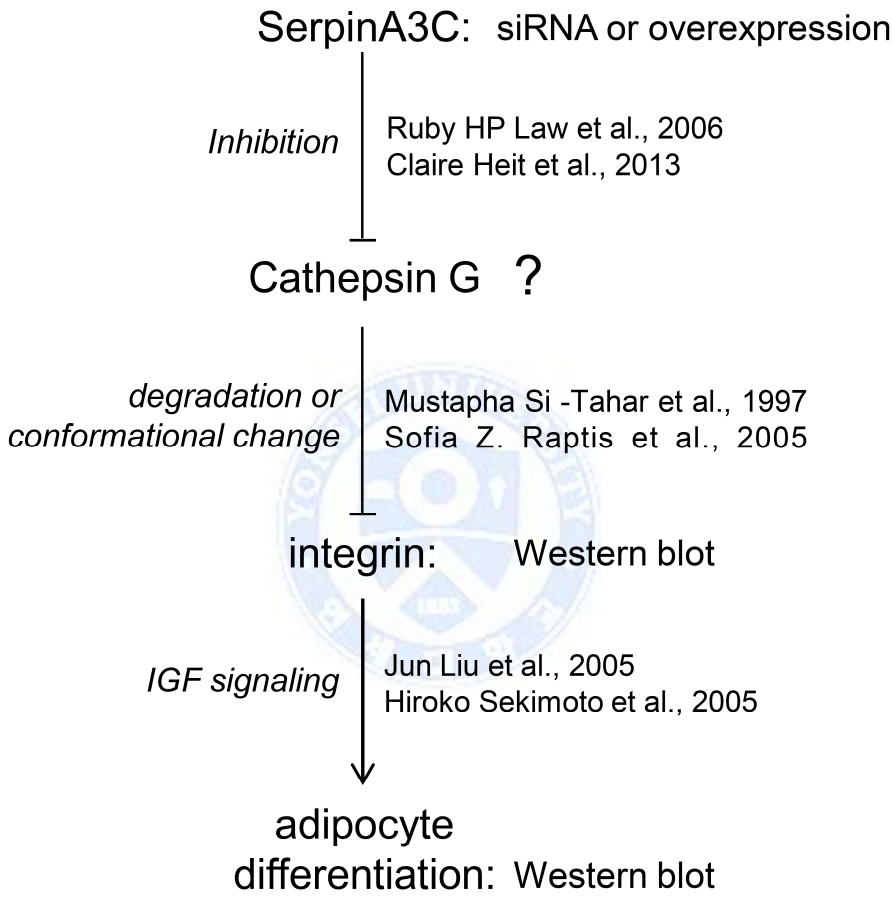
In contrast, AKT activation was prolonged in Serpina3c-knockdown cells (Fig. 9C). Phosphorylation of Glycogen synthase kinase 3β (GSK3β) was also sustained after 4 hr in knocked down cells (Fig. 9C). This data demonstrated that in Serpina3c-knockdown cells, activated AKT phosphorylates GSK3β continuously after MDI induction whereas AKT works transiently during 4 hr in control cells.

In 3T3-L1 cells, C/EBPβ is phosphorylated by two molecules, which are MAPK and GSK3β.[7,32] First phosphorylation on Thr188 by MAPK primes C/EBPβ for its subsequent phosphorylation on Ser184 and Thr179 by GSK3β. After the second phosphorylation, C/EBPβ finally acquires DNA binding affinity and plays a role as a transcription factor.[7,25]

However, phosphorylated GSK3β cannot translocate to the nuclear. In nuclear fraction of 3T3-L1 adipocytes, only total form of GKS3β was found (Fig. 9D). On the contrary, phosphorylated GSK3β was found only in cytosol (Fig. 9E). This data indicated that continuously phosphorylated GSK3β cannot phosphorylate C/EBPβ in the nucleus thus inhibited adipogenesis in Serpina3c-knockdown cells.

The expression of C/EBPα and PPARγ, which are normally used as key markers of adipogenesis, was considerably reduced in Serpina3c-knockdown cells, whereas expression of C/EBPβ was slightly decreased (Fig. 9F). Taken together, these data indicate that Serpina3c is involved in adipogenesis via IGF-1 pathway mediated by integrin α5 and β3 heterodimers.

A


SerpinA3C: siRNA or overexpression
Inhibition
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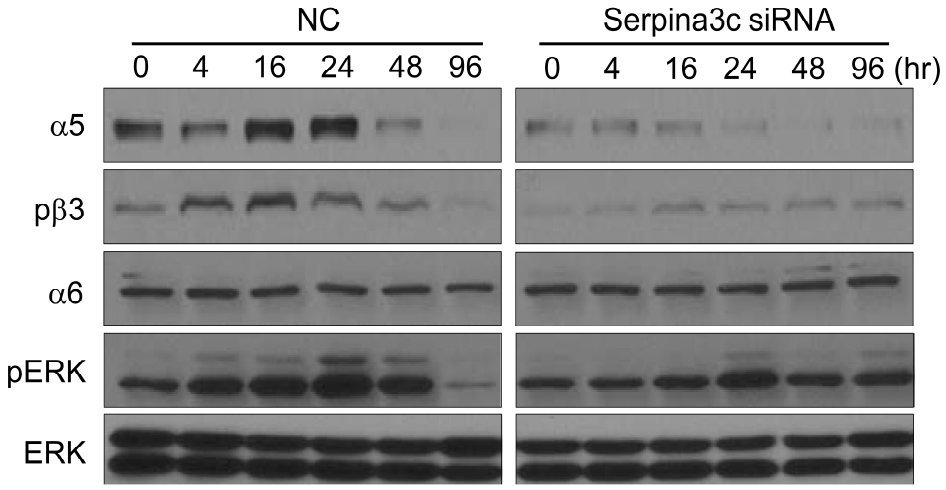
Claire Heit et al., 2013
Cathepsin G ?
degradation or conformational change
Mustapha Si -Tahar et al., 1997
Sofia Z. Raptis et al., 2005
integrin: Western blot
IGF signaling
Jun Liu et al., 2005
Hiroko Sekimoto et al., 2005
adipocyte differentiation: Western blot

B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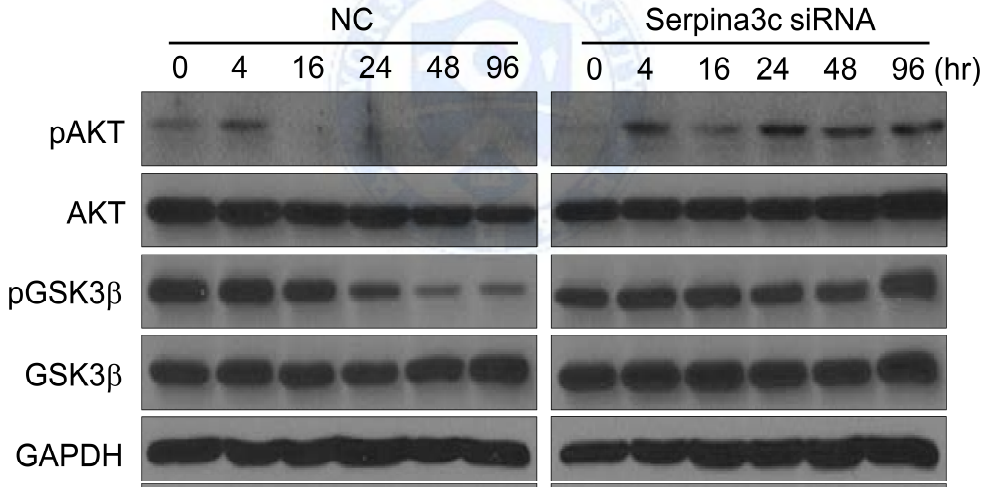
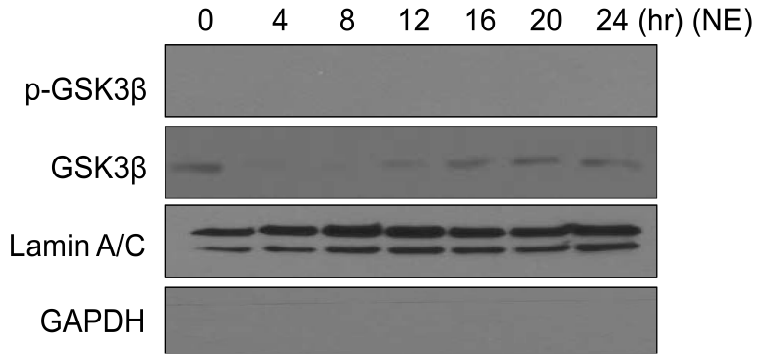
α6
pERK
ERK

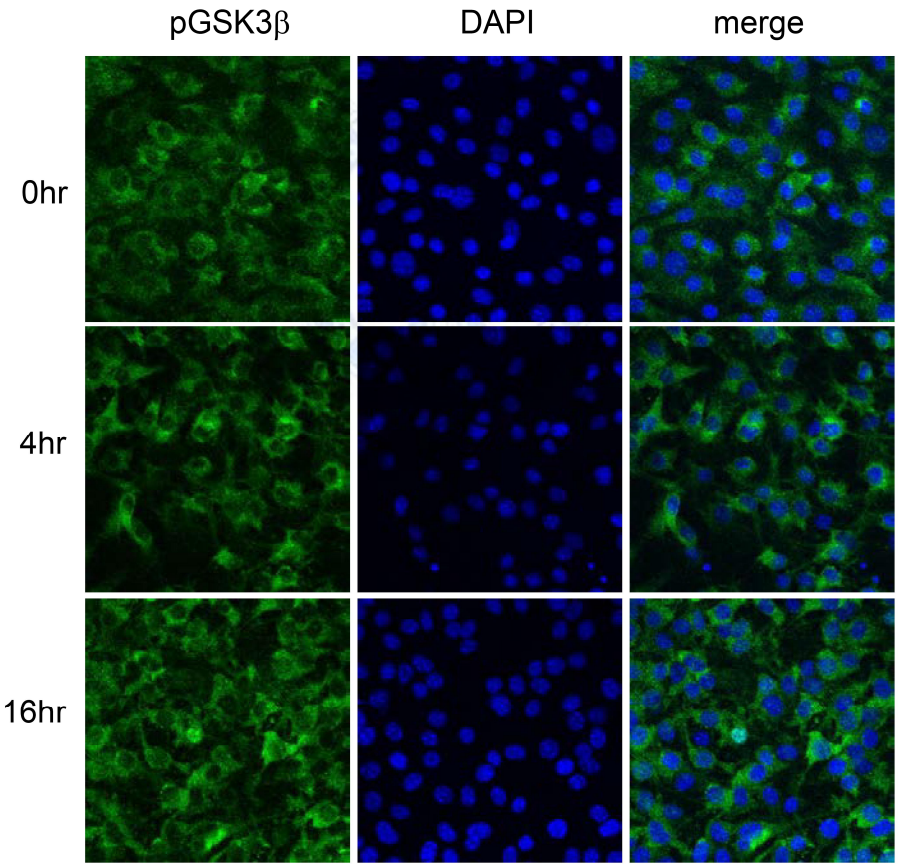
C


NC
Serpina3c siRNA
0 4 16 24 48 96
0 4 16 24 48 96 (hr)
pAKT
AKT
pGSK3β
GSK3β
GAPDH

D


0
4
8
12
16
20
24 (hr) (NE)
p-GSK3β
GSK3β
Lamin A/C
GAPDH

E


pGSK3β
DAPI
merge
0hr
4hr
16hr

F

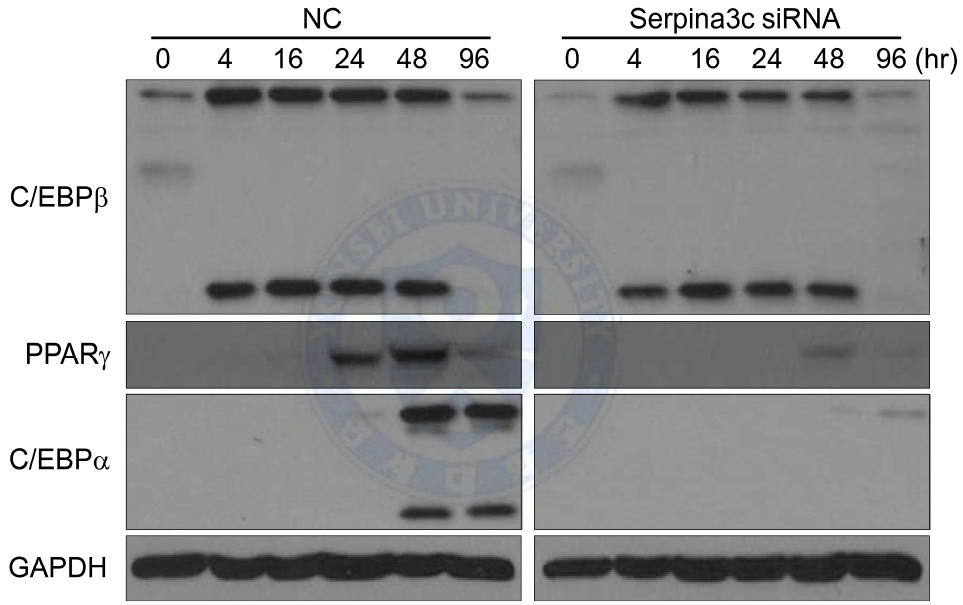

NC
Serpina3c siRNA
0 4 16 24 48 96
0 4 16 24 48 96 (hr)
C/EBPβ
PPARγ
C/EBPα
GAPDH

Figure 9. Serpina3c is required for integrin-mediated IGF-1 pathway resulting in ERK activation and AKT inactivation. (A) A schematic model of hypothesis. (B, C, F) 3T3-L1 cells were transfected with negative control (NC), or Serpina3c siRNA, and then differentiated using MDI. Cell lysates were prepared at various times after adipogenic induction. All protein levels in control cells and Serpina3c-knockdown cells were measured by western blotting, with GAPDH as a loading control. (D) After MDI induction, nuclear extract of 3T3-L1 cells was prepared at various times. pGSK3β and GSK3β levels were measured by western blotting. GAPDH and LaminA/C were used as a cytosol protein marker and nuclear protein marker respectively. (E) 3T3-L1 cells were induced to differentiate with MDI. After 4 hr and 16 hr, cells were fixed and subjected to immunofluorescence analysis with antibody against pGSK3β. Fluorescence images were obtained by confocal microscopy.

8. Overexpression of Serpina3c in 3T3-L1 preadipocytes increased adipocyte-specific gene expression.

If Serpina3c-knockdown suppresses adipogenesis, overexpression of Serpina3c will increase adipocyte differentiation. To examine this, mouse Serpina3c was cloned in pcDNA3.0 with FLAG-tag. Because Serpina3c is a secretory protein, FLAG-tag must be cloned in C-terminal of Serpina3c. After transfection and MDI induction, it was shown that C/EBPα and PPARγ were increased in western blot analysis (Fig. 10A, B). These data suggest that Serpina3c plays a critical role in 3T3-L1 adipocytes differentiation and overexpression of Serpina3c facilitates adipogenesis.

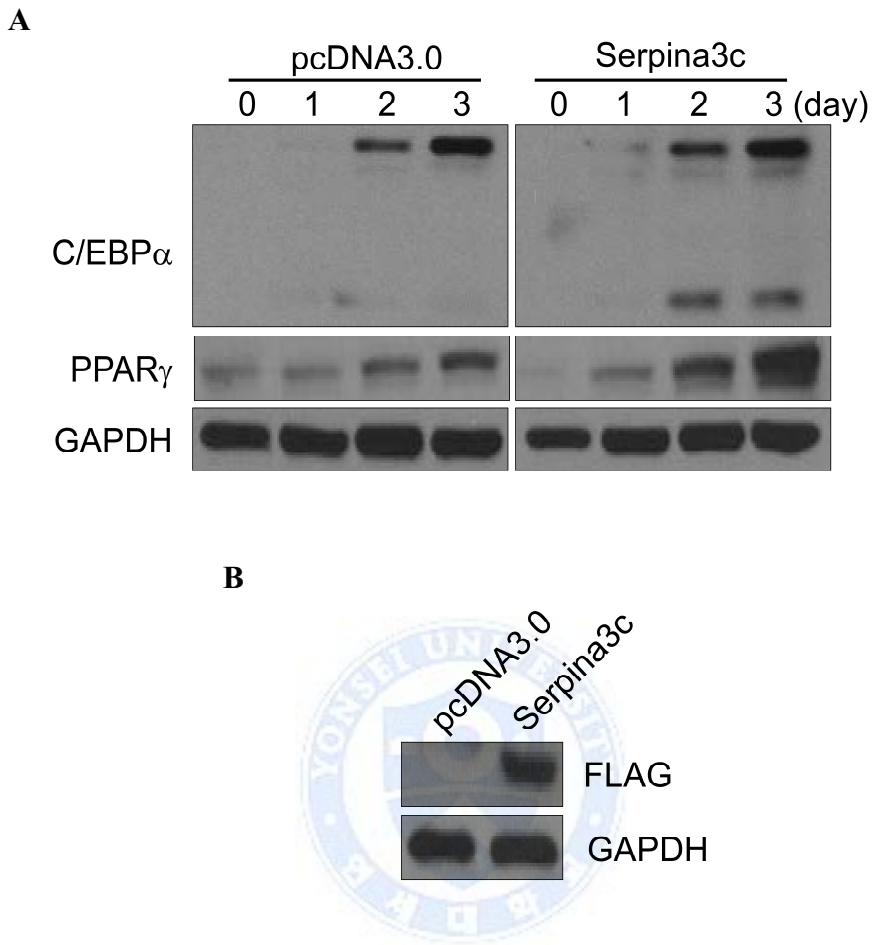


Figure 10. Overexpression of Serpina3c increased adipocyte-specific gene expression. (A) 3T3-L1 cells were transfected with expressing control vector (pcDNA3.0) or Serpina3c-cloned vector (pcDNA3.0-Serpina3c-FLAG) by electroporator and then induced to differentiation. Collected proteins at indicated time after induction were used to assay the level of C/EBPα and PPARγ by western blotting with GAPDH as a loading control. (B) Overexpressed Serpina3c was detected by FLAG-tag with whole cell lysates after two days from transfection.

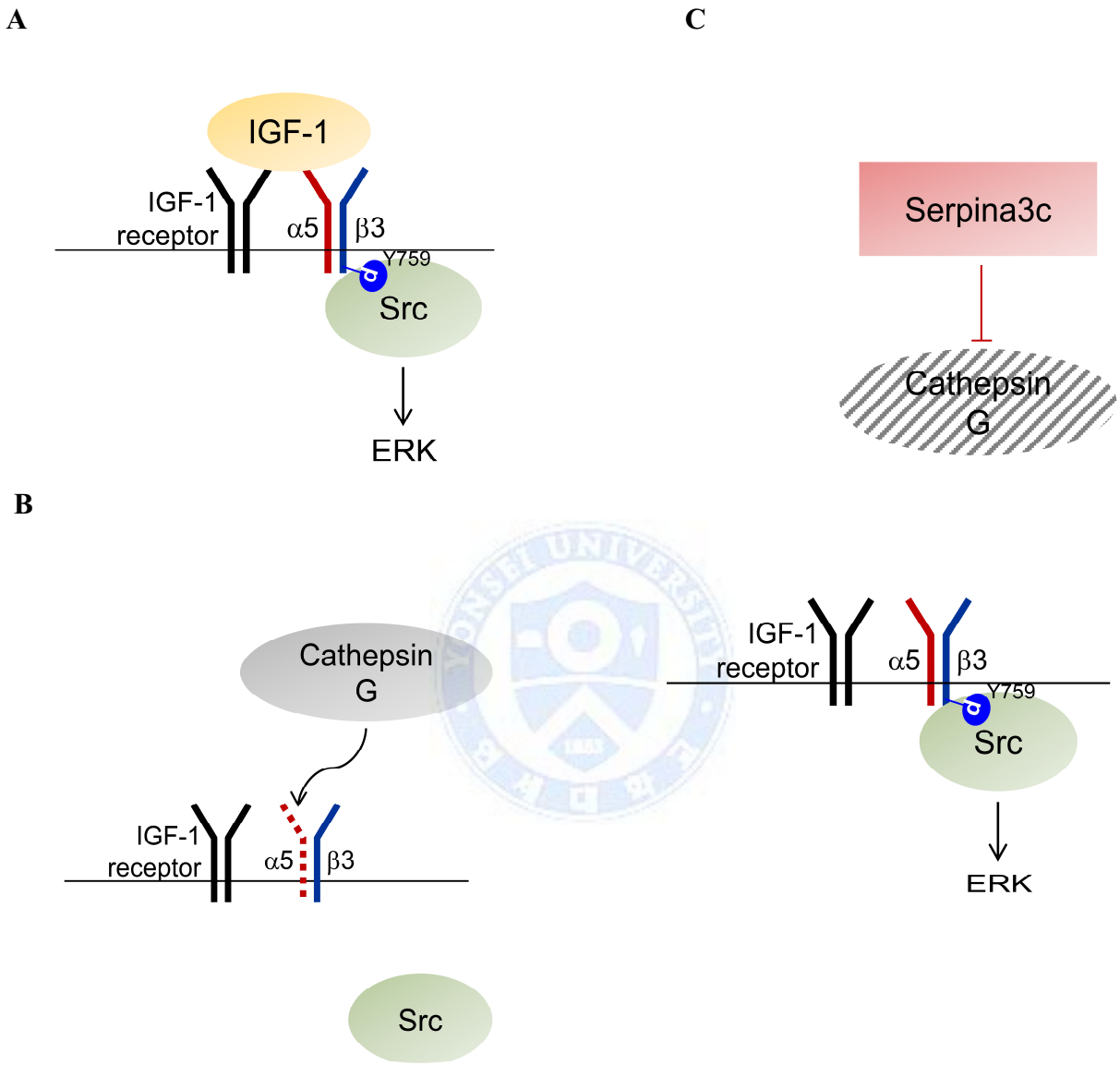

A
IGF-1
IGF-1
receptor
α5
β3
Y759
Src
ERK
C
Serpina3c
Cathepsin
G
B
Cathepsin
G
IGF-1
receptor
α5
β3
IGF-1
receptor
α5
β3
Y759
Src
ERK
Src

Figure 11. A schematic model of Serpina3c functions in adipogenesis. (A) IGF-1 receptors form a complex with integrin α5-β3 heterodimer that is required for activation of IGF-1 pathway. When the ligand binding, β3-Tyr759 is phosphorylated and this phosphorylation leads to Src activation. (B) If cathepsin G exists, cathepsin G degrades α5 that IGF-1 receptor complex is not formed. Therefore, IGF-1 cannot bind to the receptor and IGF-1 signaling is not transduced. (C) Serpina3c inhibits cathepsin G. Inhibited cathepsin G cannot degrade α5 resulting in increase of intact α5. Thus, IGF-1 receptor complexes are increased and this may lead to more IGF-1 binding and Src activation.

V. DISCUSSION

3T3-L1 cells are useful *in vitro* models to investigate developmental process of adipocytes. Since 3T3-L1 cells were cloned by Howard green,[33] there have been many efforts to elucidate the mechanisms of 3T3-L1 cells differentiation. Thanks to these efforts, it was revealed that several transcription factors, like C/EBPβ, C/EBPα, and PPARγ play a key role in adipogenesis.[5,7] However, there is a clear need to fully define the complex molecular processes that control adipocyte differentiation.

In the previous study, it was found out that CM which was collected from differentiating adipocytes accelerated other preadipocytes differentiation.[8] In this regard, it was assumed that in CM, there are some secreted molecules and these molecules stimulate unknown genes involved in adipocyte differentiation. These unknown genes may code transcription factors, secretory enzymes, or could be any other molecules related in adipogenesis. In any case, this stimulation of CM caused changes of gene-expression profile and these changes lead to facilitated differentiation. Therefore, it is possible to find out novel genes involved in adipogenesis by comparing these changes.

Using RNA sequencing, overall gene-expression profile was compared between two adipocyte groups which were CM induced adipocytes and MDI induced adipocytes. To filter out RNA sequencing data, I set up largely two criteria. First, I selected genes which are expressed more than two-fold higher in CM after 4 hr from induction or 24 hr from induction. At this point, 4 hr and 24 hr of time

points were very significant. 4 hr means the time for immediate response against CM. If the CM stimulates the expression of any genes, they will respond instantly within 4 hr when C/EBPβ starts expression. In addition, 24 hr means the time for the end of first cell cycle during mitotic clonal expansion. Because CM-induced adipocytes skip mitotic clonal expansion, they may proceed differentiation at 24 hr. On the other hand, MDI-induced adipocytes are still proliferating in mitotic clonal expansion at 24 hr. Therefore, it is possible to search novel genes involved in differentiation by comparing these two groups. Second, I selected genes which are constantly up-regulated after induction except for down-regulated genes. As a result, 51 candidate genes were screened from RNA sequencing data applying these two criteria. Although it was focused on Serpina3c in this study, the other 50 candidate genes are worthy enough to be researched further.

Using this CM system, Serpina3c was selected as a novel gene expected to be involved in adipogenesis. Serpins are the largest and the most broadly distributed superfamily of protease inhibitors.[10,34,35] Serpins appear to be ubiquitous in multicellular higher eukaryotes. In 3T3-L1 cells, other Serpins like Serpina3n, Serpine1, Serping1, Serpinf1, Serpinb8, and Serpinb9 were also expressed proven by RNA sequencing in this study. But among these Serpins, Serpina3c was the most suitable gene which was answered my criteria.

Serpin A clade, which is containing Serpina3c and Serpina3n, is classified as antitrypsin-like, extracellular proteins.[11] SerpinA3 is an inhibitory protein formerly known as an inhibitor of chymotrypsin and cathepsin G.[11,35] Serpin is normally

found in blood, liver, kidney, and lung.[11] But represented data suggest that Serpin also exists in adipose tissue in a high level (Fig. 4).

Because Serpina3c is a secretory protein, it is able to affect extracellular matrix, like integrins. In this study, it was assumed that this secretory Serpina3c may inhibit cathepsin G like other SerpinA3 clade proteins. Cathepsin G is a kind of serine protease and it is related with degradation or conformational changes of integrins in immune cells.[26,27] Therefore, if Serpina3c is knocked down in the cells, cathepsin G will be fully activated that integrin degradation will be accelarated. In the present data, integrin α5 was decreased in Serpina3c-knockdown cells (Fig. 9B). As a result, this impaired integrin α5 malfunctioned in IGF-1 pathway and affected downstream signalings such as ERK and AKT (Fig. 9B, C). This model is summarized in Fig. 10.

All the result in this study provides important advances to understanding the mechanism of adipogenesis. First, I introduced CM as a tool for searching novel genes related with 3T3-L1 differentiation. CM powerfully stimulates adipocyte differentiation without mitotic clonal expansion. Therefore it can be possible to search genes which directly trigger differentiation without mitotic clonal expansion. Furthermore, I found out the important role of extracellular matrix during adipogenesis. Integrin, a kind of extracellular matrix, have been overlooked its role in adipogenesis. My data showed that decrease of integrin α5 induces abnormal IGF-1 signaling (Fig. 9B, C). These results indicated that integrin has a critical role in adipocyte differentiation by mediating IGF-1 signaling.

IV. CONCLUSION

In summary, the data presented in this reports suggest that conditioned medium accelerated the differentiation into mature adipocytes. Using CM system, I found out that Serpina3c is required for adipogenesis playing an important role in the transition from mitotic clonal expansion to terminal differentiation. In addition, Serpina3c in involved in adipogenesis via AKT and ERK signaling mediated by integrinα5.

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Abstract (IN KOREAN)

Conditioned medium을 이용한 지방 전구 세포 초기 분화에 결정적 영향을 미치는 새로운 유전자의 발굴 및 기능 분석

< 지도교수 김재우 >

연세대학교 대학원 의과학과

최윤정

3T3-L1 지방전구세포에 3-isobutyl-1-methylxanthine, dexamethasone, insulin (MDI) 을 처리하면 분화가 시작된다. MDI 처리 이후, 세포들은 분화를 진행하며 여러 가지 물질들을 배지 내로 분비하는데, 이렇게 세포가 분비한 미지의 물질들이 포함된 배지를 조정배지 (CM) 라고 하였다. 본 연구진의 선행연구를 통하여, 조정배지를 다른 지방전구세포에 처리하면 MDI 를 처리했을 때보다 분화가 촉진된다는 사실이 밝혀졌다. 이 사실에

근거하여, 조정배지 내에는 분화를 촉진하는 물질들이 포함되어 있을 것으로 기대하고 3T3-L1 분화에 관여하는 새로운 유전자들을 찾기 위하여 본 연구를 시작하였다. 후보 유전자들을 탐색하기 위해 먼저 CM 과 MDI 로 각각 분화 유도된 지방세포들을 대상으로 RNA sequencing 을 수행하였다. 도출된 RNA sequencing 결과 중, 분화 유도 후 CM 에서 더 높게 발현된 유전자들만을 선별하여 51 개의 후보 유전자를 선정하였다. 이 유전자들의 단백질 코딩 유무와 세포 내에서의 기능 등을 고려하여 다시 10 개의 유전자로 후보 범위를 좁혔고, 이 10 개의 유전자들을 대상으로 3T3-L1 세포와 마우스 지방 조직에서의 실제 발현량을 확인하였다. 그 결과, Serpina3c, Serpina3n, Adamts15, 세 개의 유전자로 범위가 압축되었고, 이 유전자들을 대상으로 한 RNA interference study 결과로 Serpina3c 가 최종 후보 유전자로 선정되었다. Serpina3c 는 serine protease inhibitor A3C 의 약자로, Serpin superfamily 의 한 종류이다. 3T3-L1 세포에서 Serpina3c 의 발현을 억제시켰을 때, mitotic clonal expansion 이 G_1 기에 정체되는 모습을 보였다. 특히 주목할만한 점은 Serpina3c 의 발현 억제시, integrin α5 와 인산화된 β3 가 감소하였다는 것이다. 그 결과, ERK pathway 의 활성은 감소하고 AKT pathway 의 활성은 증가하였는데, 이는 Serpina3c 는 integrin 을 매개로 한 IGF-1 신호전달체계에 관여한다는 점을 시사한다. 3T3-L1 세포에서 Serpina3c 를 과발현하면 지방세포로의 분화가 촉진되어 지방세포 특이 유전자인

C/EBPα와 PPARγ의 발현이 증가하였다. 결과적으로 Serpina3c 는 지방형성에서 중요한 인자로 작용하며, mitotic clonal expansion 과 integrin 매개의 IGF-1 신호 전달 체계에 관여하여 지방 분화와 중성지방 축적에 요구되어짐을 발견하였다.

핵심되는 말 : 지방분화, 3T3-L1,조정배지, Serpna3c, integrin